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Homologous Recombination Rescues Mismatch-Repair-Dependent Cytotoxicity of S_N1-Type Methylating Agents in *S. cerevisiae*

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Summary

Resistance of mammalian cells to S_N1-type methylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) generally arises through increased expression of methylguanine methyltransferase (MGMT), which reverts the cytotoxic O^6-methylguanine (^{Me}G) to guanine, or through inactivation of the mismatch repair (MMR) system, which triggers cell death through aberrant processing of ^{Me}G/T mispairs generated during DNA replication when MGMT capacity is exceeded [1]. Given that MMR and ^{Me}G-detoxifying proteins are functionally conserved through evolution, and that MMR-deficient *Escherichia coli dam*$^-$ strains are also resistant to MNNG [2], the finding that MMR status did not affect the sensitivity of *Saccharomyces cerevisiae* to MNNG [3] was unexpected. Because ^{Me}G residues in DNA trigger homologous recombination (HR) [4–7], we wondered whether the efficient HR in *S. cerevisiae* might alleviate the cytotoxic effects of ^{Me}G processing. We now show that HR inactivation sensitizes *S. cerevisiae* to MNNG and that, as in human cells, defects in the *MMR* genes *MLH1* and *MSH2* rescue this sensitivity. Inactivation of the *EXO1* gene, which encodes the only exonuclease implicated in MMR to date [8, 9], failed to rescue the hypersensitivity, which implies that scExo1 is not involved in the processing of ^{Me}G residues by the *S. cerevisiae* MMR system.

Results and Discussion

In the yeast *Saccharomyces cerevisiae*, the functional homolog of human MGMT, scMgt1p, has been shown to remove methyl groups from ^{Me}G residues and, to a lesser extent, from O^4-methylthymine [10], and to protect the cells from both mutagenesis and killing induced by MNNG [3]. The key players in postreplicative MMR are the MutS homologs scMsh2p, scMsh3p, and scMsh6p and the MutL homologs scMlh1p and scPms1p (functional homolog of the human PMS2). These polypeptides are also functionally highly related to the human proteins, yet the MMR status of *S. cerevisiae* was reported not to influence the response of *mgt1* mutants to methylating agents [3]. This difference between the lower and higher eukaryotic cells is unlikely to be due to the lack of apoptosis in yeast because mammalian cells can be efficiently killed by MNNG without having to activate the machinery of programmed cell death [11] and because even *dam*$^-$ MMR-deficient *E. coli* are more resistant to killing by this methylating agent than MMR-proficient strains [2]. We therefore reasoned that the different response of yeast and mammalian cells to methylating agents might be explained either by differences in DNA-damage recognition or else by differences in other pathways of methylation-damage processing.

The human mismatch binding factor hMSH2/hMSH6 (hMutSα) has been reported to bind oligonucleotide substrates containing ^{Me}G/T or ^{Me}G/C mispairs [12], and we wanted to test whether the yeast proteins behaved similarly. To this end, we overexpressed the *S. cerevisiae* MMR-recognition factor scMsh2p/scMsh6p (scMutSα) and purified it to near homogeneity (Figure 1A). Gel-shift assays with increasing amounts of scMutSα (1.7–67 nM) and a constant amount (6.6 nM) of ^{Me}G/T-, ^{Me}G/C-, G/T- and G/C-containing oligonucleotide duplexes (Figures 1B–1E) revealed that all DNA substrates formed slowly migrating protein/DNA complexes at high (>30 nM) scMutSα concentrations. Formation of these nonspecific (ns) complexes has been observed previously [13] and probably results from aggregation of scMsh2p/scMsh6p heterodimers on the same oligonucleotide substrate. In contrast, the ^{Me}G/T and G/T substrates formed a more rapidly migrating specific (s) complex with the heterodimer; this complex already appeared at low protein concentrations and represented ~90% of the total labeled oligonucleotide duplex at 16.4 nM protein concentration (lane with an asterisk in Figures 1B and 1C). In case of the ^{Me}G/C substrate, only a small amount of the specific complex was formed at lower protein concentrations (~35% at 16.4 nM scMutSα, Figure 2E, lane with an asterisk), but this amount was higher than that formed by the G/C homoduplex substrate (~10%, see Figure 2D, lane with an asterisk). In both latter cases, the nonspecific band was also apparent at the 16.4 nM scMutSα concentration, indicating a weak recognition of the ^{Me}G/C substrate. These results were confirmed in competition assays, in which the labeled G/T heteroduplex (6.6 nM) in the presence of scMutSα (16.4 nM) was competed with a 10-fold excess of the unlabeled ^{Me}G/T, ^{Me}G/C, G/T, or G/C duplexes. In these experiments, the ^{Me}G/T oligonucleotide appeared to be an even better substrate for scMutSα than G/T (Figure 1F), although it should be pointed out that the affinity of the mismatch binding heterodimer for the methylated oligonucleotides is highly dependent on sequence context, which is generally not the case for G/T (our unpublished observations). Differences in DNA-damage recognition thus cannot explain the difference in phenotype between yeast and mammalian cells. We therefore argued that these differences must lie in the processing of methylation damage downstream from damage recognition.

Treatment of mammalian and yeast cells with MNNG was reported to give rise to elevated levels of homologous recombination (HR). Furthermore, in human cells,

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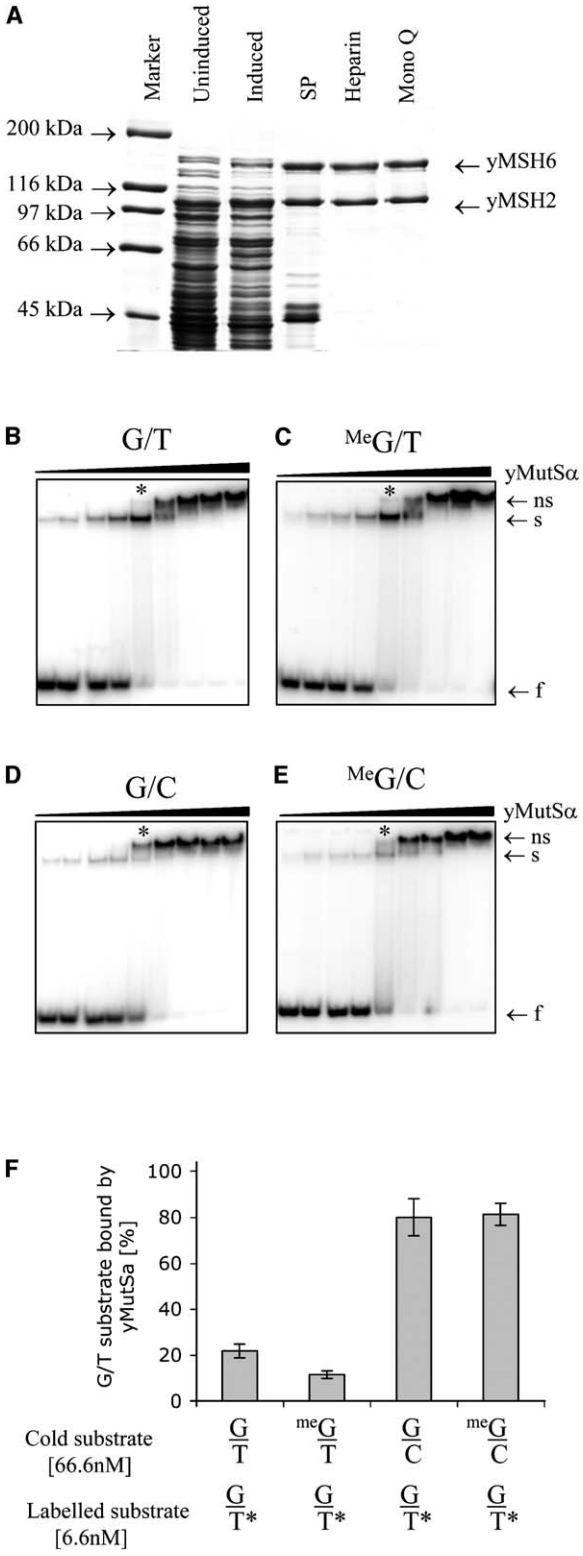

Figure 1. Substrate Specificity of the *S. cerevisiae* Mismatch Binding Factor Msh2p/Msh6p

(A) Overexpression of scMsh2p/Msh6p in *E. coli*. Uninduced, total extract from BL21 codon plus cells; Induced, total extract from BL21 codon plus cells overexpressing scMsh2p/Msh6p; SP, Heparin and Mono Q, pooled fractions eluted from the respective FPLC chromatography columns. The figure shows aliquots from different purification steps separated on a denaturing 7.5% polyacrylamide gel stained with Coomassie Blue.

(B–E) Binding of the scMsh2p/Msh6p heterodimer to different DNA substrates. Formation of specific (s) and nonspecific (ns) complexes. The heterodimer (1.7–67 nM) was incubated with ^{32}P-labeled 6.6 nM G/T, ^{Me}G/T, G/C, or ^{Me}G/C DNA substrates. The lane with 16.4 nM protein concentration is indicated by an asterisk. The protein/DNA complexes were analyzed by a gel-shift assay as described in the Experimental Procedures and visualized by autoradiography. (E) Competition binding assay. The Msh6p/Msh2p heterodimer (16.4 nM) was incubated with 6.6 nM ^{32}P-labeled G/T heteroduplex. The preformed complexes were then challenged with a 10-fold excess of unlabeled G/T, ^{Me}G/T, G/C, and ^{Me}G/C DNA substrates. The fraction of the labeled G/T substrate bound by Msh2p/Msh6p was quantitated by Typhoon 9600 PhosphoImager with ImageQuant software.

HR was elevated specifically in an MMR-dependent manner [4–7]. Given that the efficiency of HR in *S. cerevisiae* is substantially higher than in mammalian cells, we wondered whether this could be the underlying cause of the different responses of these organisms to methylating agents. Inactivation of the *RAD52* gene, which is required for most HR processes in *S. cerevisiae*, rendered the cells hypersensitive to MNNG. Additional inactivation of the *MMR* genes *MSH2* or *MLH1* had no effect on sensitivity, which was not particularly surprising given that these cells expressed scMgt1p (Figure 2A). However, when the *MGT1* gene was also inactivated, the *mgt1 rad52* double mutant became exquisitely sensitive to MNNG, whereas the *mgt1 rad52 msh2* and *mgt1 rad52 mlh1* triple mutants were sensitized to a substantially lesser extent (Figure 2B). It therefore appears that MMR-mediated processing of ^{Me}G residues gives rise to cytotoxic intermediates that are resolved by homologous recombination. Due to the high efficiency of HR in yeast, these intermediates are most likely successfully repaired, which would account for the substantial resistance of MMR-proficient yeast cells to MNNG. Interestingly, the survival curve of the *mgt1 rad52* strain appears to be biphasic (Figure 2B). At low MNNG concentrations (0.5–1.5 μM), the inactivation of *MSH2* or *MLH1* fully suppresses the sensitivity of the *mgt1 rad52* strain to MNNG, which shows that the killing is at this concentration range linked almost exclusively to the processing of ^{Me}G residues by the MMR system. In contrast, cell death at high MNNG concentrations (>3 μM) is also most likely caused by other types of damage, such as strand breaks arising through processing of *N*-methylated purines and abasic sites, which account for more than 90% of the damage caused by these agents. Moreover, overexpression of scMgt1p in the *rad52* strain failed to improve survival at high MNNG concentrations (data not shown), which further supports the hypothesis that the cytotoxicity is in this case linked to DNA modifications distinct from ^{Me}G. A similar situation was also observed in human cells [14].

It is well established that both scMsh2p and scMlh1p are absolutely required for MMR, whereas the mechanism and players in the downstream events of the repair process remain enigmatic. We thus decided to examine the involvement of the *EXO1* gene, which en-

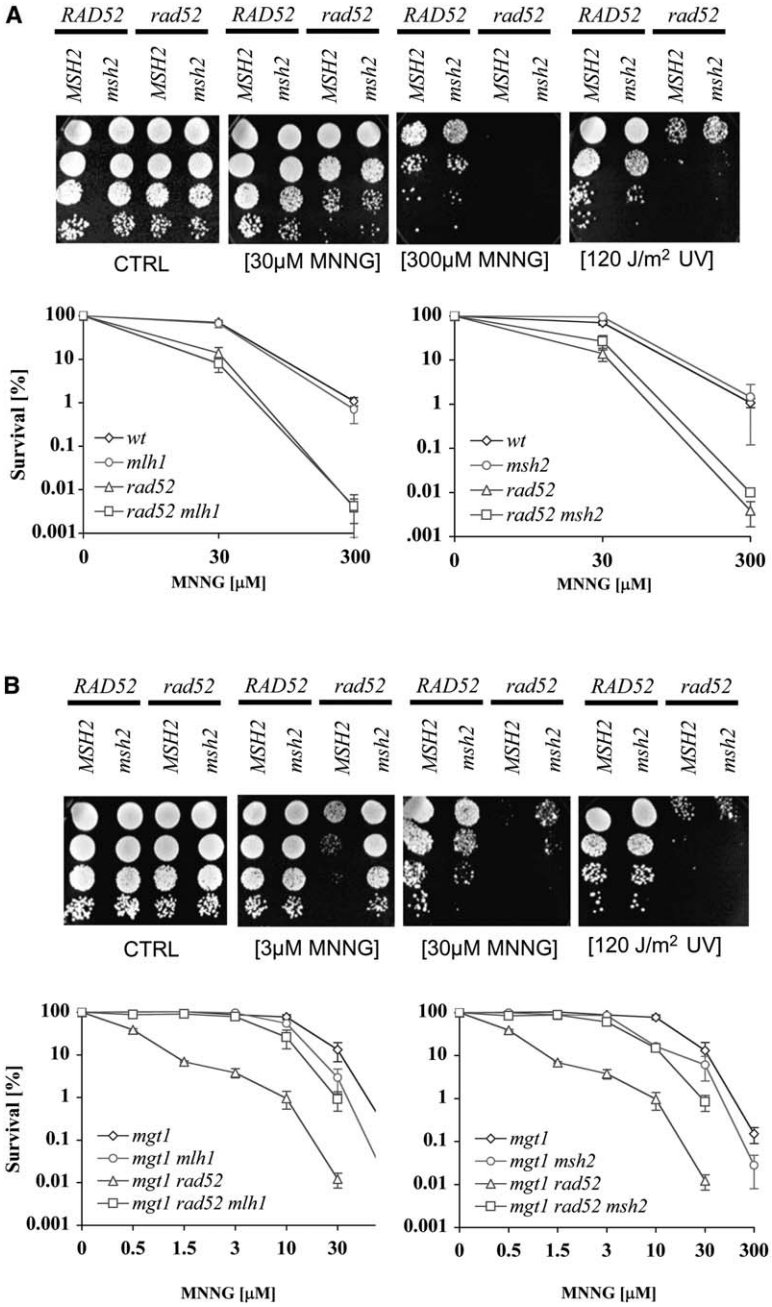

Figure 2. MNNG-Induced Killing of *S. cerevisiae* Strains with Different Genetic Backgrounds

(A) Comparison of MNNG sensitivities of the MMR-deficient (*mlh1* or *msh2*) and/or recombination-deficient (*rad52*) strains as measured by the spot test. Mid-log phase cells were treated with the indicated concentrations of MNNG, harvested and spotted on YPD plates at proper serial dilutions as described in the Experimental Procedures. (Wild-type genes are labeled with capitals, deletion mutants with lowercase letters) Sensitivity to ultraviolet (UV) light was used as a control. The upper panel shows a representative experiment, and the lower panel shows a graphic representation of data pooled from 3–5 independent experiments. Error bars show standard error of the mean.

(B) Effect of methylguanine methyltransferase (*mgt1*) deficiency on the sensitivity of MMR- and/or recombination-deficient strains to MNNG. Sensitivity to UV light was used as a control. The upper panel shows a representative experiment, and the lower panel shows a graphic representation of data pooled from 3–5 independent experiments. Error bars show standard error of the mean.

codes the only exonuclease implicated in MMR to date (with the notable exception of the proofreading activity of polymerase delta [8, 15]) in the processing of methylation damage induced by MNNG. In contrast to *MSH2* and *MLH1* inactivation, which rescued the hypersensitive phenotype of the *mgt1 rad52* mutant strain, deletion of the *EXO1* gene brought about a further, albeit minor, increase in MNNG sensitivity (Figure 3). These results suggest that scExo1p helps the cell overcome the deleterious effects of DNA methylation, rather than being involved in the MMR-dependent cytotoxic processing of ^{Me}G residues. The role of the scExo1p in MMR has been the subject of some discussion. The protein plays a role in several other biological processes, including mutation-avoidance pathways distinct from MMR, telomere integrity, and processing of double-strand breaks prior to homologous recombination, and it is likely that its functions overlap with those of other exonucleases [16]. Although our data provide evidence that scExo1p is not required for the processing of methylation damage, they fail to indicate which exonuclease (if any) fulfills this role in vivo.

The interaction between yeast MMR and HR in the processing of DNA damage has been described previously [17]. In that study, disruption of *MMR* genes conferred a mild but significant (1.5- to 6-fold) resistance to cisplatin, carboplatin, and doxorubicin, and contrary to our results, the resistant phenotype was de-

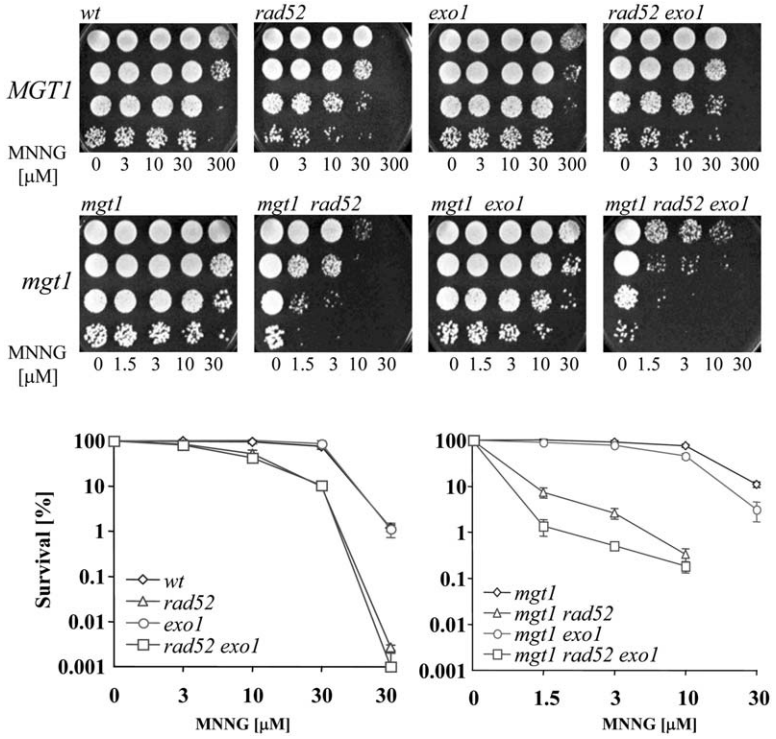

Figure 3. MNNG Sensitivities of Recombination- (*rad52*), Methylguanine Methyltransferase- (*mgt1*), and/or Exonuclease 1 (*exo1*)-Deficient *S. cerevisiae*

Mid-log phase cells of the indicated strains were treated with the indicated concentrations of MNNG and spotted at proper serial dilutions on YPD plates as described in the Experimental Procedures. The upper panel shows a representative experiment, and the lower panel shows a graphic representation of data pooled from 3–5 independent experiments. Error bars show standard error of the mean.

pendent on functional scRad52p. This difference can be explained. DNA lesions induced by the above agents block DNA replication and require recombination for efficient lesion bypass [17]. Because MMR controls the fidelity of recombination processes, it most likely lowers recombination frequency in DNA containing these bulky adducts and thus augments cytotoxicity. In contrast, ^{Me}G/C pairs arising in methylguanine-methyltransferase-deficient cells upon methylation do not block DNA synthesis per se and are well tolerated in the absence of MMR. Our model for the cytotoxicity of S_N1-type methylating agents is outlined in Figure 4. In the absence of MMR, DNA replication will give rise to one unmodified progeny DNA molecule and one that carries the ^{Me}G residue paired either with thymine of cytosine, neither of which needs to be resolved by recombination. In the presence of MMR, the ^{Me}G/C or ^{Me}G/T mispairs arising during replication are detected by the scMsh2/scMsh6 heterodimer, which triggers a round of repair. However, because MMR is directed to the newly synthesized DNA strand, the modified nucleotide persists in the template strand. Because the polymerase filling the repair patch cannot find a perfect base-pairing partner for ^{Me}G, it may leave a gap or a similar lesion that could be repaired by HR. In the absence of HR, the gap would be converted during the next replication round to a double-strand break that might trigger cell-cycle arrest and cell death.

In summary, we show that damage reversal by scMgt1p, along with methylation damage processing and repair by HR, mask the sensitivity of MMR-proficient *S. cerevisiae* cells toward killing by S_N1-type methylating agents. Inactivation of MMR in the *mgt1 rad52* yeast background rendered cells approximately 20-fold more tolerant to killing by MNNG Recombination-deficient *S. cerevisiae* thus resemble the methylation-sensitive phenotype of mammalian cells [18]. The lack of involvement of scExo1p in the processing of methylation damage implies that the exonuclease function is fulfilled by another enzyme(s). Given the amenability of *S. cerevisiae* to genetic manipulation and high-throughput screening, our present results should help us design assays for identification of these enzymes, as well as of other gene products involved in the processing of methylation damage. It is hoped that at least some of these findings will help us understand the mode of action of S_N1 methylating agents, which represent an important class of cancer chemotherapeutics.

Experimental Procedures

Production of scMsh2-scMsh6 in *E. coli*

The pET11a-scMSH2-scMSH6 plasmid (a kind gift of M. Hingorani) was transformed into BL21 DE3 codon plus cells (Strategene). The induction and purification was performed essentially as described [19], except that ultrasonic treatment was used for cell disruption.

Oligonucleotide Substrates

The 34-mers 34TopG (AATTCCCGGGGATCCGTCGACCTGCAGCC AAGCT), 34Top^{Me}G (AATTCCCGGGGATCCGTC^{Me}GACCTGCAGC CAAGCT), 34BottomT (AGCTTGGCTGCAGGTTGACGGATCCCCG GGAATT) and 34BottomC (AGCTTGGCTGCAGGTCGACGGATCC CCGGGAATT) were synthesized by Microsynth (Balgach, Switzerland) and purified by polyacrylamide gel electrophoresis (the sites of mismatch or base modification are underlined). Despite the fact that Microsynth strictly adhered to the protocol for ^{Me}G incorporation into the oligonucleotide, as recommended by the manufacturer of the corresponding phosphoramidite (Glenn Research, USA), mass-spectrometric analysis by NanoESI revealed that a substantial part of the ^{Me}G-containing oligonucleotide still contained the

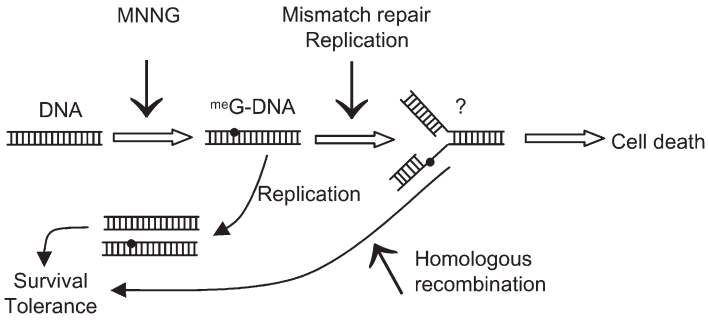

Figure 4. Model of MMR-Induced Killing upon Treatment with Methylating Agents

See text for details.

isobutyryl protecting group on the exocyclic nitrogen of the methylated guanine. The fully deprotected oligonucleotide had to be separated from the contaminant by reverse-phase HPLC, for which a Nucleosil 100Å, C_{18}, 5 μm, 250 × 4.0 mm column (BGB Analytik) eluted with a linear gradient of 5%–20% acetonitrile in 100 mM triethylamonium acetate (pH 7) was used. The "bottom" oligonucleotides were radioactively labeled with [γ-^{32}P]-ATP (Amersham Biosciences) and polynucleotide kinase (New England Biolabs) and purified on Sephadex G25 columns. Three pmol of the labeled "bottom" oligonucleotides were then annealed with 4.5 pmol of the cold top oligonucleotides in 1× polynucleotide kinase buffer by brief heating to 95°C and slow cooling to room temperature. In this way we obtained the duplex substrates G/C, G/T, ^{Me}G/C, and ^{Me}G /T.

Gel-Shift Experiments

The indicated amounts of purified *S. cerevisiae* Msh2p/Msh6p protein (diluted where necessary in 50 mM NaCl, 25 mM Tris.HCl [pH 8.0]) and 6.6 nM of the labeled substrate in binding buffer (10% glycerol, 0.33 mg/ml BSA, 25 mM HEPES [pH 8.0], 0.5 mM EDTA, and 0.5 mM DTT) were incubated for 20 min at room temperature in a total volume of 30 μl. The reaction products were separated on 6% polyacrylamide gels (acrylamide:bisacrylamide ratio 19:1) at room temperature at 150V for 85 min. The gels were dried and scanned with the Typhoon 9400 imager and ImageQuant TL software (both Amersham Biosciences).

Yeast Media

For unselective growth, YPD medium (2% glucose, 2% bactopeptone, and 1% yeast extract) was used. Clones where the gene of interest was replaced by the KANMX cassette were selected on YPD plates supplemented with 200 μg/ml G418 (Invitrogen). Where necessary, the media were solidified by 2% agar (Difco). All yeast strains were propagated under aerobic conditions at 30°C.

Yeast Strains and Transformation

The yeast strains used in this study were all isogenic derivatives of FF18733 and FF18734 *S. cerevisiae* strains (a kind gift of F. Fabre)

Table 1. *S. cerevisiae* Strains Used in This Study

Strain	Relevant Genotype	Source
FF18733	MATa; leu2-3, 112; ura3-52; his7-2; lys1-1, trp1-289	F. Fabre
ZS30	FF18733 with *msh2::KANMX*	Z. Storchova
EP82	FF18733 with *mlh1::KANMX*	E. Papouli
FPC 45	FF18733 with *rad52::URA3*	this study
FPC 1-1	FF18733 with *mgt1::KANMX*	this study
FPC 50	FF18733 with *exo1::KANMX*	this study
FPC 32	FF18733 with *mgt1::KANMX; rad52::URA3*	this study
FPC 24	FF18733 with *rad52::URA3; msh2::KANMX*	this study
FPC 37	FF18733 with *rad52::URA3; mlh1::KANMX*	this study
FPC 3-3b	FF18733 with *mgt1::KANMX; msh2::KANMX*	this study
FPC15	FF18733 with *mgt1::KANMX; mlh1::KANMX*	this study
FPC 52	FF18733 with *rad52::URA3; exo1::KANMX*	this study
FPC 55	FF18733 with *mgt1::KANMX; exo1::KANMX*	this study
FPC 21	FF18733 with *mgt1::KANMX; rad52::URA3; msh2::KANMX*	this study
FPC 61	FF18733 with *mgt1::KANMX; rad52::URA3; exo1::KANMX*	this study
FPC 39	FF18733 with *mgt1::KANMX; rad52::URA3; mlh1::KANMX*	this study
FF18734	MATα; leu2-3, 112; ura3-52; his7-2; lys1-1, trp1-289	F. Fabre
ZS30-1d	FF18734 with *msh2::KANMX*	this study
EP 85	FF18734 with *mlh1::KANMX*	E. Papouli
FF18743	FF18734 with *rad52::URA3*	F. Fabre
FPC 2-1	FF18734 with *mgt1::KANMX*	this study
FPC 51	FF18734 with *exo1::KANMX*	this study
FPC 30	FF18734 with *mgt1::KANMX; rad52::URA3*	this study
FPC 28	FF18734 with *rad52::URA3; msh2::KANMX*	this study
EP 95	FF18734 with *rad52::URA3; mlh1::KANMX*	E. Papouli
FPC 3-2a	FF18734 with *mgt1::KANMX; msh2::KANMX*	this study
FPC 16	FF18734 with *mgt1::KANMX; mlh1::KANMX*	this study
FPC 54	FF18734 with *rad52::URA3; exo1::KANMX*	this study
FPC 58	FF18734 with *mgt1::KANMX; exo1::KANMX*	this study
FPC 22	FF18734 with *mgt1::KANMX; rad52::URA3; msh2::KANMX*	this study
FPC 59	FF18734 with *mgt1::KANMX; rad52::URA3; exo1::KANMX*	this study
FPC 43	FF18734 with *mgt1::KANMX; rad52::URA3; mlh1::KANMX*	this study

and are listed in Table 1. Replacement of the *MMR* genes was performed with *kanMX4* replacement cassettes with specifically designed primers (see below), with pUG6 (*MSH2*, *MGT1*, and *EXO1*) or pFA6a-*kanMX4* (*MLH1*) plasmids being used as templates for polymerase chain reactions (PCR), essentially as described [20]. The primer sequences for gene disruption were as follows (forward, reverse primer):

MSH2, GACACTCTACTCCAATATCAACTGTAAAAAATCTCTTTATCTGCTGGACCTAACATCAAAATCCTCAGATTAAAAGGAGCTGAAGCTTCGTACGC, CTTTCCAATGCATATTATATGTACTATTTGTATCTATATATTATCTATCGATTCTCACTTAAGATGTCGTTGTAATATTAATTATAACAACGCATAGGCCACTAGTGGATCTG;

MLH1, ATAGTGATAGTAAATGGAAGGTAAAAATAACATAGACCTATCAATAAGCACAGCTGAAGCTTCGTACGC, AAAGGAAAGGGCATACACTTTCAAATGAAACACAATCACACTCAGGAAATGCATAGGCCACTAGTGGATCTG;

MGT1, TGGCAGGGCATTTAAAATGCGGTGGAAACAAGGAAGATTAATCAAGTAATGATATAGCATCAGCTGAAGCTTCGTACGCTGCAG, CAATTTACCACATATACATAACTATTTCTTATGTTTATTTTCCTAAAATCCTTTATCCAAGCATAGGCCACTAGTGGATCTG;

EXO1, TGCTTTTTGGACCACATTAAAATAAAAGGAGCTCGAAAAAACTGAAAGGCGTAGAAAGGACAGCTGAAGCTTCGTACGCTGCAG,TTCGACGAGATTTTCATTTGAAAAATATACCTCCGATATGAAACGTGCAGTACTTAACTTGCATAGGCCACTAGTGGATCTG.

The transformations were performed by the lithium-acetate method. The genotypes of all strains used were verified by PCR (primer sequences and details are available on request), Southern blotting, and tetrad analysis.

Spot Tests

A stock solution of 1 M MNNG (Sigma) was prepared in DMSO and stored in the dark at –20°C. Because of the estimated ~45 min half-life of MNNG in aqueous solutions, all experiments were performed in liquid cultures as follows: The cells were inoculated from a YPD plate into 3 ml of liquid YPD medium and cultivated overnight. The cells were then diluted 1:15 with YPD, and 3 ml cultures were incubated for a further 3 hr, when the cells were again in an exponential growth phase. The cells were then mock-treated and treated with several concentrations of freshly diluted MNNG for 45 min. They were harvested, washed, and spotted (~12 μl drops) at serial dilutions on YPD plates. For UV treatments, the cells from the untreated cultures were spotted at similar dilutions on several YPD plates and subsequently irradiated with the indicated doses (UV Stratalinker 1800). The plates were evaluated after 3 days of cultivation at 30°C. Because no significant differences were observed between the a and α mating types, the results shown are based on 3–5 independent experiments carried out with strains in both a and α backgrounds.

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